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Nitrogen loss caused by denitrifying *Nitrosomonas* cells using ammonium or hydrogen as electron donors and nitrite as electron acceptor

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Abstract Cells of the obligately lithotrophic species *Nitrosomonas europaea* and *Nitrosomonas eutropha* were able to nitrify and denitrify at the same time when grown under oxygen limitation. In addition to oxygen, nitrite was used as an electron acceptor. The simultaneous nitrification and denitrification resulted in significant formation of the gaseous N-compounds nitrous oxide and dinitrogen, causing significant nitrogen loss. In mixed cultures of *N. europaea* and various chemoorganotrophic bacteria, the nitrogen loss was strongly influenced by the partners growing under oxygen limitation. Under anoxic conditions, pure cultures of *N. eutropha* were able to denitrify with molecular hydrogen as electron donor and nitrite as the only electron acceptor in a sulfide-reduced complex medium. The increase of cell numbers was directly coupled to nitrite reduction. Nitrous oxide and dinitrogen were the only detectable end products. In pure cultures of *N. eutropha* and mixed cultures of *N. eutropha* and *Enterobacter aerogenes,* ammonium and nitrite disappeared slowly at a molar ratio of about one when oxygen was absent. However, under these conditions cell growth was not measurable.

Key words *Nitrosomonas* · Lithotrophic and mixotrophic nitrification · Oxygen limitation · Aerobic and anaerobic denitrification · Nitrous oxide · Dinitrogen Hydroxylamine · Hydrogen · Ammonium

Introduction

Nitric oxide, nitrous oxide, and dinitrogen are well-documented gaseous products of the lithotrophic ammonia-oxidizer *Nitrosomonas* (Hooper 1968; Poth and Focht 1985; Poth 1986). The enzyme nitrite reductase is involved in N_2O production (Hooper 1968), whereas NO seems to be generated mainly by chemodenitrification of nitrite (Stüven et al. 1992). Nitrite reductase activity is induced by lowering the oxygen partial pressure (Miller and Nicholas 1985). Considering that lithotrophic ammonia-oxidizers can reduce nitrite with hydroxylamine (Hooper 1968), it is possible that these organisms are able to carry out all reduction steps from nitrite to dinitrogen. It is not known whether this reaction is restricted to oxic conditions. In anoxic wastewater reservoirs, Abeliovich (1987) had shown a significant increase in cell numbers of *Nitrosomonas* in the hypolimnion. No explanation had been given for this observation.

Nitrification and denitrification are important in biological wastewater treatment systems. The existence of oxic/anoxic interfaces in activated sludge flocs (Klapwijk 1985) supports the hypothesis that the production of gaseous N-compounds observed in the presence of oxygen is explained by conventional denitrification. However, Robertson et al. (1988) has given evidence that *Paracoccus denitrificans (= Thiosphaera pantotropha)* is an aerobic denitrifier and a heterotrophic nitrifier. In contrast, the facultative nitrifier *Nitrobacter* is also an anaerobic denitrifier (Bock et al. 1988).

So far, cell growth of *Nitrosomonas* by denitrification has not been reported. The aim of this work was to understand the mechanism of nitrogen loss by production of gaseous N-compounds in pure cultures of *Nitrosomonas europaea* and *Nitrosomonas eutropha* and in mixed cultures with other organisms.

Materials and methods

Organisms

Experiments under oxic conditions were performed with *Nitrosomonas europaea* strain "Freitag" (isolated from a sewage pipe of the Hamburg sewer system) and *Nitrosomonas eutropha* strain 904 (isolated from cattle manure); experiments under anoxic conditions were performed with *Nitrosomonas eutropha.* The species were identified according to Koops et al. (1991). The *Nitrosomonas* strains are kept in the culture collection of the Institut für Allge-

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meine Botanik (University of Hamburg). In mixed culture experiments, the following chemoorganotrophic bacteria were used: *Paracoccus denitrificans* (= *Thiosphaera pantotropha* DSM 2944T; LMD 82.5), *Pseudomonas aeruginosa*, *Alcaligenes eutrophus*, *Acinetobacter calcoaceticus*, *Enterobacter aerogenes*, *Hydrogenophaga pseudoflava*, *Pseudomonas saccharophila*, *Ancylobacter aquaticus*.

Media

For lithotrophic growth of *Nitrosomonas*, the following medium was used: distilled water (1 l), NH_4Cl (535 mg), NaCl (585 mg), $CaCl_2 \cdot 2H_2O$ (147 mg), KCl (74 mg), KH_2PO_4 (54 mg), $MgSO_4 \cdot 7H_2O$ (49 mg), $FeSO_4 \cdot 7H_2O$ (0.973 mg), H_3BO_3 (0.049 mg), $MnSO_4 \cdot H_2O$ (0.045 mg), $ZnSO_4 \cdot H_2O$ (0.043 mg), $(NH_4)_6Mo_7O_{24} \cdot 7H_2O$ (0.037 mg), $CuSO_4$ (0.016 mg), cresol red (5 mg), Hepes (19.064 g). The medium was adjusted to pH 7.0 and sterilized at 110°C for 1 h.

The mineral medium was supplied with the following organic compounds: bactopeptone (150 mg), trisodium citrate dihydrate (40 mg), sodium acetate (40 mg), and sodium lactate (40 mg). This medium was called synthetic wastewater.

Experiments under anoxic conditions were performed in a modified medium for methanogenic bacteria according to Whitman et al. (1992). For mixotrophic growth conditions, the following medium was used: distilled water (1 l), yeast extract (1.5 g), tryptic soy broth (1.5 g), resazurin (1 mg), sodium acetate (5 g), Na_2S (0.5 g), NH_4Cl (0.5 g), $MgSO_4 \cdot 7H_2O$ (1.0 g), $CaCl_2$ (0.4 g), $K_2HPO_4 \cdot 3H_2O$ (0.4 g), nitrilotriacetic acid (15 mg), $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (2 mg), Na_2SeO_3 (2 mg), $CoCl_2 \cdot 6H_2O$ (1 mg), $MnSO_4 \cdot 2H_2O$ (2 mg), $Na_2MoO_4 \cdot 2H_2O$ (1 mg), $Na_2WO_4 \cdot 2H_2O$ (1 mg), $ZnSO_4 \cdot 7H_2O$ (1 mg), $AlCl_3 \cdot 6H_2O$ (0.4 mg), $NiCl_2 \cdot 6H_2O$ (0.25 mg), H_3BO_3 (0.1 mg), $CuSO_4 \cdot 5H_2O$ (0.1 mg), 4-aminobenzoic acid (0.1 mg), nicotinic acid (0.1 mg), Ca-pantothenate (0.1 mg), pyridoxine-hydrochloride (0.1 mg), riboflavin (0.1 mg), thiamine hydrochloride (0.1 mg), biotin (0.05 mg), folic acid (0.05 mg), lipoic acid (0.05 mg), vitamin B_{12} (0.05 mg). The apparent redox potential of the medium was –250 to –300 mV. Organic compounds were excluded from the medium for experiments under lithotrophic growth conditions.

Growth conditions

Stock cultures of *Nitrosomonas* were kept in 1-l Erlenmeyer flasks with 600 ml of synthetic wastewater medium at 28°C in the dark. The cultures were neither stirred nor shaken. Oxygen was supplied by diffusion from the surface of the medium. Chemoorganotrophic bacteria were grown in synthetic wastewater medium at 28°C in the dark.

Pure cultures of *N. europaea* and *N. eutropha* and mixed cultures of *N. europaea* and various chemoorganotrophic bacteria were grown at 28°C in the dark in 1-l Erlenmeyer flasks containing 600 ml of synthetic wastewater medium. Some experiments were performed in a 2.5-l bioreactor equipped with an automatic control system for pH, PO_2, and temperature (Meredos, Nörten-Hardenberg, Germany). This apparatus was used to keep a constant PO_2 within *Nitrosomonas* cultures.

Anaerobic pure cultures of *N. eutropha* were grown in 50 ml modified medium for methanogens (Whitman et al. 1992) under mixotrophic and lithotrophic conditions in 100-ml serum bottles sealed with rubber stoppers. The cultures were kept under an atmosphere of H_2/CO_2 (80:20, v/v) at 28°C in the dark.

N. eutropha was grown in pure and mixed culture with *Enterobacter aerogenes* in 50 ml synthetic wastewater medium in 100-ml serum bottles sealed with rubber stoppers. Anoxic conditions were achieved by suffocation. After 50-days' incubation, oxygen was consumed.

Analytical procedures

Cell numbers were determined by light microscopy with the aid of a Helber chamber. The determination of nitrogenous compounds except hydroxylamine was carried out according to Stüven et al. (1992). Hydroxylamine was determined according to Fiadeiro et al. (1967) after ion exchange with Amberlite CG400II (Serva, Heidelberg, Germany). Electron microscopy techniques were those described by Bock and Heinrich (1969). Hydrogenase activity was measured according to Reh and Schlegel (1981). Dinitrogen formation was established by use of a gas chromatograph (SiChromat 2, Siemens, Karlsruhe, Germany) with thermal conductivity detection.

The gas chromatography column (3 m · 0.32 cm) was filled with a molecular sieve (5 Å, 80–100 mesh). Helium served as carrier gas. Oxygen partial pressure was measured with a Clark-type oxygen electrode. Anoxic conditions were controlled by using the redox indicator resazurin.

Results

Nitrification and denitrification under oxygen limitation

In pure cultures of *Nitrosomonas europaea*, about 19% of the ammonium-N provided is converted to gaseous N-compounds when the cells are grown in the presence of organic matter at a reduced oxygen partial pressure (Stüven et al. 1992). When *N. europaea* was grown mixotrophically in synthetic wastewater in a series of four subcultures, up to 36% of ammonia-N was lost by the production of gaseous end products. More nitrogen was lost as the number of inoculation steps increased. In these experiments, the cells were grown in 1-l Erlenmeyer flasks containing 600 ml medium. The oxygen supply was diffusion-limited. When 10 mM ammonium was consumed, sedimented cells were used to inoculate subcultures into fresh synthetic wastewater medium. The influence of low

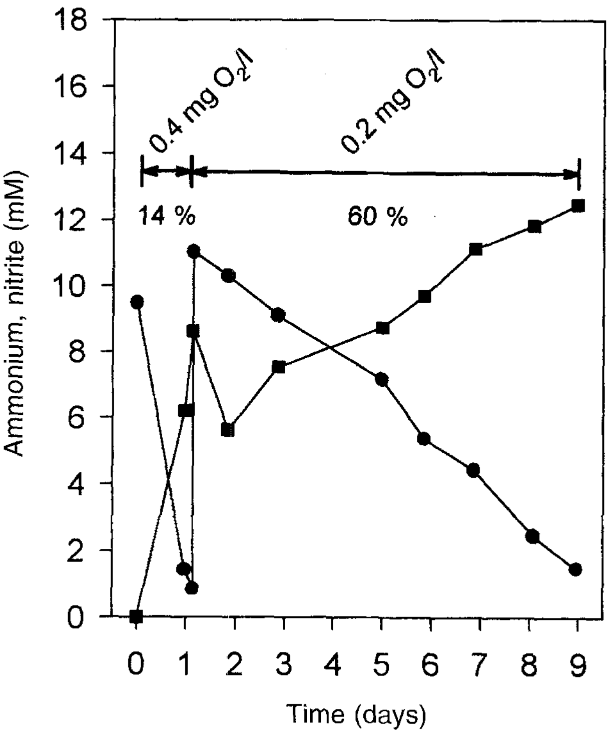

Fig. 1 Induction of *Nitrosomonas* cells by oxygen limitation during growth in synthetic wastewater medium. Oxygen concentration and nitrogen losses (percent of ammonium-N not converted to nitrite) are indicated. After 30 h, ammonium was added again. *Filled circles* ammonium, *filled squares* nitrite

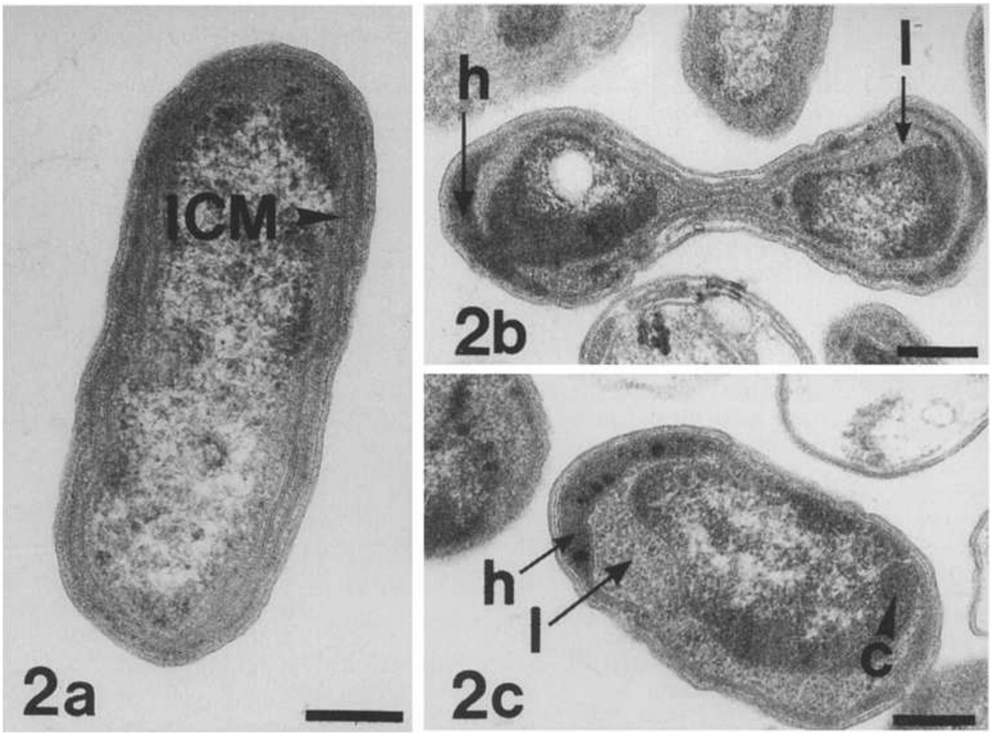

Fig. 2 Electron micrographs of ultrathin sections of **a** nitrifying and **b** denitrifying cells of *Nitrosomonas europaea* and **c** denitrifying cells of *N. eutropha*. Nitrifying cells were grown with ammonium under high oxygen partial pressure, while denitrifying cells were grown with ammonium and organic material under oxygen limitation. In denitrifying cells, the layers between the intracytoplasmic membranes (*ICM*) were filled with material of high electron density (*h*) and low electron density (*l*)

Table 1 Hydroxylamine formation in anoxic and oxic cultures of *Nitrosomonas europaea* (oxic) and *Nitrosomonas eutropha* (anoxic). In controls without organisms, hydroxylamine was not detectable (*M* methanogen medium, *S* synthetic wastewater medium)

Growth conditions	Number of parallels	Medium	NH_2OH (μM)
Oxic	3	S	0.3–3
Anoxic	9	S	0.3–1.2
Anoxic	10	M	0.3–0.7

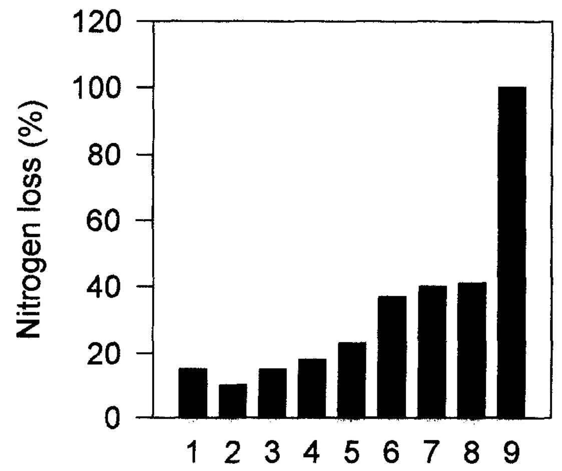

Fig. 3 Comparison of the nitrogen losses in pure cultures of *Nitrosomonas europaea* and in mixed cultures with various chemoorganotrophic bacteria in synthetic wastewater medium under oxygen limitation. After growth, 10 mM ammonium was consumed. *1* Pure culture of *Nitrosomonas europaea*, *2–9* mixed cultures of *N. europaea* with the following chemoorganotrophic bacteria: *2 Pseudomonas saccharophila*, *3 Enterobacter aerogenes*, *4 Ancylobacter aquaticus*, *5 Acinetobacter calcoaceticus*, *6 Hydrogenophaga pseudoflava*, *7 Alcaligenes eutrophus*, *8 Pseudomonas aeruginosa*, *9 Paracoccus denitrificans*

oxygen partial pressure could be demonstrated in an experiment performed in a bioreactor with oxygen control (Fig. 1). After lowering the oxygen concentration from 0.4 to 0.2 mg/l, a significant decrease of ammonia oxidation activity was observed and the amount of nitrogen lost increased from 14 to 60% (Fig. 1). *N. europaea* cells and *N. eutropha* cells were able to adapt to the low oxygen partial pressure (data not shown). When grown under oxygen limitation for several months, they retained and even enhanced their ability to reduce nitrite and cause nitrogen loss. These cells were called denitrifying cells.

Using transmission electron microscopy (TEM), ultrathin sections of adapted *N. europaea* and *N. eutropha* cells were shown to differ from those grown with sufficient aeration (Fig. 2). In contrast to the densely stacked intracytoplasmic membranes (ICM) of well-aerated nitrifying cells of *N. europaea* (Fig. 2a) and *N. eutropha* (not shown), the distances between the membrane layers of denitrifying cells had increased. Between these membranes, layers of high electron density were distinguishable from those of low electron density (Fig. 2b, c). When grown with ammonium in the presence of organic matter, the cells were still able to eliminate ammonium nitrogen even under lithotrophic conditions when the oxygen supply was diffusion-limited. Well-aerated subcultures did not produce N_2O or N_2. The ultrastructure of these cells no longer differed from lithoautotrophically grown cells of *N. europaea* and *N. eutropha* (not shown).

Cells oxidizing ammonium and simultaneously reducing nitrite under oxygen limitation excreted hydroxy-

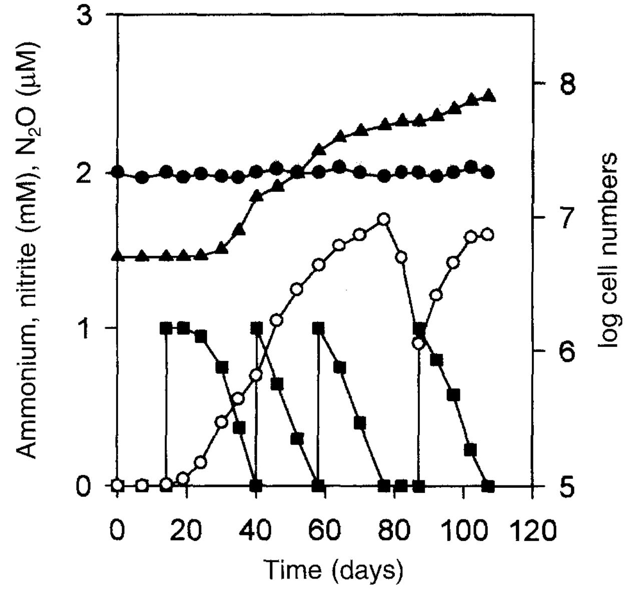

Fig. 4 Anaerobic growth of a pure culture of *Nitrosomonas eutropha* with molecular hydrogen and nitrite with negative (–250 to –300 mV) apparent redox potential. N_2O was detected as an intermediate. *Filled circles* ammonium, *filled squares* nitrite, *open circles* N_2O, *filled triangles* cell numbers

lamine within a concentration range of 0.3–3 µM, indicating that hydroxylamine is an intermediate of ammonia oxidation. Even under anoxic conditions, hydroxylamine was detectable in various media (Table 1).

In oxygen-limited mixed cultures of *N. europaea* or *N. eutropha* with various chemoorganotrophic bacteria, more nitrogen was lost than in pure cultures (Fig. 3). In mixed cultures of *N. europaea* or *N. eutropha* with *P. denitrificans,* no nitrite was detected after 10 mM NH_4^+ was oxidized. In contrast to pure cultures of each of the *Nitrosomonas* species, the loss of more than two-thirds of the ammonium nitrogen is probably due to the activity of the aerobic denitrifier *P. denitrificans.*

Since a measured concentration of 10 µM nitrous oxide (N_2O) in the headspace of the flasks and a theoretical concentration of 66 µM N_2O dissolved in the medium was far too low to explain the loss of dissolved N-compounds, dinitrogen is proposed to be the major gaseous end product.

Denitrification under anoxic conditions

Under anoxic conditions, molecular hydrogen served as the only electron donor for nitrite reduction provided that the apparent redox potential of the medium was low (–250 to –300 mV). As demonstrated in Fig. 4, added nitrite was consumed immediately in the presence of H_2, while ammonium was not oxidized. Hydrogenase activity was detectable in *N. eutropha* cells and in *N. europaea* cells and was much higher in cells of *N. eutropha.* Cell growth of *N. eutropha* was directly coupled to nitrite reduction. Again, only small amounts of N_2O and NH_2OH were detected (Fig. 4, Table 1). Dinitrogen was formed as the main end product. After consumption of 1.8 mM nitrite, 1.1 mM dinitrogen was detected in the headspace by GC analysis. This value is almost in accordance with the theoretical value (0.9 mM). In the absence of hydrogen, under an atmosphere of argon or dinitrogen with organic matter as the only electron donor, nitrite reduction activity decreased about fivefold and cell growth was slow (not shown).

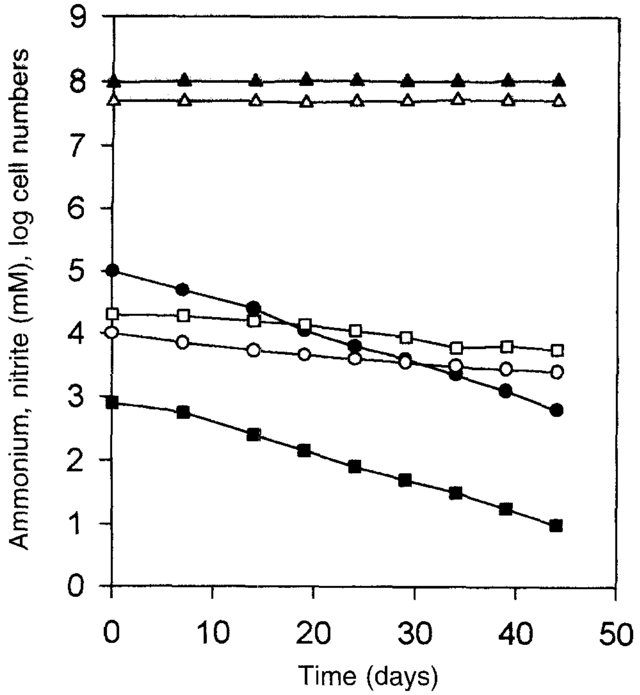

Fig. 5 Simultaneous elimination of ammonium and nitrite in anoxic pure cultures of *Nitrosomonas eutropha* and in mixed culture with *Enterobacter aerogenes* in synthetic wastewater medium. Mixed culture of *E. aerogenes* and *N. eutropha*: *filled circles* ammonium, *filled squares* nitrite, *filled triangles* cell numbers. Pure culture of *N. eutropha*: *open circles* ammonium, *open squares* nitrite, *open triangles* cell numbers

Under anoxic conditions achieved by suffocation, even ammonium was a suitable electron donor for nitrite reduction. In a mixed culture of $1 \cdot 10^8$ *N. eutropha* cells per ml and $1 \cdot 10^4$ *E. aerogenes* cells per ml, 2.2 mM ammonium was consumed within 44 days, but no increase in cell numbers was detected (Fig. 5). In pure cultures of *N. eutropha* and in mixed cultures, ammonium and nitrite were eliminated simultaneously at a molar ratio of about one (Fig. 5). As *Enterobacter* did not oxidize ammonium nor reduce nitrite to gaseous end products, we propose that *Nitrosomonas* is responsible for the reaction described. The specific activity was much higher in mixed cultures than in pure cultures.

Discussion

Physiologically, ammonia-oxidizers of the genus *Nitrosomonas* are much more versatile than expected. At a reduced oxygen partial pressure, *N. europaea* and *N. eutropha* were able to use ammonium as electron donor and simultaneously oxygen and nitrite as electron acceptors. As the oxygen concentration decreased, more nitrite was used instead of oxygen.

Under anoxic conditions, nitrite was the only electron acceptor, and molecular hydrogen or organic compounds were suitable electron donors and were able to support cell growth. It seems likely that the nitrite reductase characterized by Hooper (1968) is part of the denitrification process with traces of nitrous oxide and dinitrogen as the main gaseous end products. Direct evidence for N_2 production was first given by Poth (1986). Further ^{15}N-experiments are in progress to show N_2 production by denitrifying *Nitrosomonas* cells.

The mechanism of nitrite reduction is obvious if molecular hydrogen (H_2) or organic material is the electron donor. *N. europaea* and *N. eutropha* both were shown to use H_2 as a substrate. Furthermore, pyruvate supports slow anaerobic growth (Abeliovich and Vonshak 1992). Following the equation proposed by Broda (1977), the use of ammonium as electron donor may have led to the production of N_2:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O; \qquad \Delta G_0' = -360\,\text{kJ/mol}$$

According to the results presented, equimolar amounts of ammonium and nitrite are quantitatively converted to molecular nitrogen. Here, we have shown for the first time that anaerobic ammonium oxidation exists in pure and mixed cultures of two *Nitrosomonas* species. The mechanism of this reaction deserves further investigation. In accordance with physiological changes, ultrastructural alterations were found. Cell growth could not be expected since the substrate consumed was much too low to support growth of a dense cell suspension.

In the presence of ammonium and organic matter more nitrogen was lost, correlating with adaptation of the *Nitrosomonas* cells to low oxygen partial pressure. These results can be explained by conventional nitrification and denitrification sequences. When grown under oxygen limitation, ammonium-oxidizing cells had layers of high and low electron density between the intracytoplasmic membranes. The layers of high electron density are proposed to contain high amounts of unidentified enzymes. In mixed cultures, the nitrogen balance was strongly influenced by the partners present. Aerobic denitrifiers and *Nitrosomonas* species in mixed culture eliminated almost all the ammonium nitrogen present. It is not yet known why *Enterobacter*, as a partner of *Nitrosomonas* species, enhanced anaerobic ammonium oxidation significantly, while *Escherichia coli* and *Serratia marcescens* did not.

Elimination of nitrogenous compounds by simultaneous nitrification and denitrification leading to a production of N_2O and N_2 is of great economic interest for wastewater treatment plants. Mulder (European Patent 0327184 A1) recently described anaerobic ammonium oxidation in wastewater; however, it is not clear which organisms are involved. Experiments using ^{15}N and enzyme studies are necessary to obtain a better insight into the complex cooperative effects in mixed cultures of ammonia-oxidizers and chemoorganotrophic bacteria.

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